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Influence of two functional polymorphisms in *NOS1* on baseline cortisol and working memory in healthy subjects

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ABSTRACT

Introduction: The neuronal isoform of the nitric oxide synthase (NOS-I) encoded by *NOS1* is the main source of nitric oxide (NO) in the brain. Reduced NO signaling in the prefrontal cortex has been linked to schizophrenia and cognitive processes while reduced striatal *NOS1* expression has been associated with impulsive behavior.
Methods: To evaluate the effect of two functional polymorphisms in alternative first exons of *NOS1*, ex1f-VNTR and ex1c-SNP rs41279104, on the HPA stress axis and neurocognitive abilities, 280 healthy subjects were genotyped, had their salivary cortisol levels measured and were assessed in verbal memory, verbal fluency, working memory and verbal IQ by using the California Verbal Learning Test (CVLT), the Regensburger test of verbal fluency (RWT), a n-back task and subscales of the Wechsler Adult Intelligence Scale III (WAIS-III).
Results: Schizophrenia risk (A)-allele carriers of *NOS1* ex1c-SNP rs41279104 displayed significantly lower baseline cortisol levels ($p = 0.004$). *NOS1* ex1f-VNTR genotype carriers showed differences in working memory performance ($p = 0.05$) in a gene-dose effect manner, with homozygous carriers of the short impulsivity-risk allele committing most commission errors. Finally, A-allele carriers of the *NOS1* ex1c-SNP rs41279104 tended to react faster during the working memory task ($p = 0.065$).
Conclusion: For the first time, we demonstrated an influence of the *NOS1* ex1c-SNP rs41279104 on salivary cortisol levels and additionally implicate the A-allele in an enhanced reaction time during a working memory task. Regarding the *NOS1* ex1f-VNTR our study supports the previously reported influence on impulsivity, lending further support to the hypothesis that this genetic variant underlies impulsive behavior.

1. Introduction

Nitric oxide (NO) acts both as a first and second messenger in numerous pathways. It therefore has many target structures: the most important one being soluble G-cyclase, but also including proteins that it nitrosylates and thereby functionally modifies [1] such as the N-methyl-D-aspartate (NMDA) receptor and dopamine and serotonin transporters [2]. NO thus acts as a link between the glutamatergic and monoaminergic pathways. Mechanistically, it is involved in long term potentiation [3] and in cGMP dependent neuronal processes including synaptic plasticity and neuronal differentiation [4].

A significant role of NOS-I in the neuroendocrine-immune axis response to stress has been suggested by Bilbo et al. who reported higher baseline cortisol levels and dampened stress-induced increases in cortisol in *NOS1* knockout mice as compared to wild type mice [5]. Complementing these findings, we have previously reported an upregulation of the glucocorticoid receptor and blunted stress response in knockdown mice [6]. However, contrasting findings in rats suggest a bidirectional involvement of NOS-I in the regulation of hypothalamic pituitary adrenal (HPA) axis activity [7].

In line with its manifold biological functions, NO has been suggested in a variety of mental disorders [8]. Reduced NO signaling in the prefrontal cortex is associated with schizophrenia and cognition while reduced *NOS1* expression in the striatum goes along with impulsive behaviors. This study addresses the question of whether two functional polymorphisms in the promoter regions of two alternative first exons in *NOS1*, ex1f-VNTR and ex1c-SNP rs41279104, influence salivary cortisol and the cognitive subsections verbal memory, verbal fluency, working

Abbreviations: ACTH, adrenocorticotrophic hormone; CVLT, California Verbal Learning Test; HPA, hypothalamic pituitary adrenal; IL-1b, Interleukin-1b; NMDA, N-methyl-D-aspartate; NOS, nitric oxide synthase; RWT, Regensburger verbal fluency test; SNP, single nucleotide polymorphism; VNTR, variable number of tandem repeats; WAIS, Wechsler Adult Intelligence Scale

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memory and verbal intelligence in healthy subjects. Both alternative exons 1c and 1f are expressed in human brain with exon 1c being expressed mostly in cortex and hippocampus [9] and exon 1f predominantly in the striatum [10].

The *NOS1* SNP rs6490121 was significantly associated with schizophrenia in a genome wide association study [11] and then followed up for its functional consequences. Homozygous carriers of the risk allele, patients as well as healthy subjects, showed poorer outcomes in tests for verbal IQ and for working memory [12], which are characteristically impaired in patients suffering from schizophrenia. The authors suggested the short allele of the *NOS1* ex1f-VNTR, which is in linkage disequilibrium (D‘ = 0.70, r^2 = 0.26, HapMap CEU) with rs6490121, as a putative functional mechanism of cognitive performance dysregulation. This length polymorphism of the *NOS1* ex1f-VNTR has been previously dichotomized into short (S; 180–196 repeats) and long (L; 198–210 repeats) alleles with the short allele being associated with a reduced expression of the *NOS1* gene as compared to the long version [10,13]. Multiple functional consequences in humans of this polymorphism have been reported including impulsivity [13–16] and specific personality traits [17] as well as a greater disease severity and impaired prefrontal brain functioning in patients suffering from schizophrenia [10]. A recent study by Rovný et al. [18] found that risk variants of ex1f-VNTR and rs6490121 were associated with a weaker prepulse inhibition (PPI) of the acoustic startle reflex in healthy subjects thus linking both variants to a well-established endophenotype of schizophrenic psychoses. In the present study, we also examined a further polymorphism which reduces the promoter activity of *NOS1* exon 1c [19]. We [20] have previously studied the effect of this variant on *NOS1* expression in post-mortem brain samples of schizophrenic patients and found that the risk allele significantly decreases expression of *NOS1* in the prefrontal cortex, possibly contributing to schizophrenia liability. In patients suffering from schizophrenia, the *NOS1* ex1c-SNP rs41279104 risk allele is associated with slower reaction time in the 2-back task which tests working memory, as well as with reduced right-hemispheric activation of the frontal cortex during a verbal fluency task [21]. Controls however showed equally long reaction times for both genotype groups in the 2-back task.

Taking into account previous research we formed three hypotheses: First, carriers of the short allele of ex1f-VNTR will show higher impulsivity which may affect their test results. Second, risk (A)-allele carriers of ex1c-SNP rs41279104 will perform worse at tasks that challenge executive functions. And third, risk allele carriers of either polymorphism will show higher baseline cortisol levels.

2. Methods

2.1. Subjects

Altogether, 280 healthy subjects of Caucasian origin participated. They were recruited by notice board and by a local website. Mental and physical health was verified via an extensive structured interview [22] and the subjects' self-disclosure. All participants gave written consent after oral as well as written explanation about the investigation we enrolled. The study was approved by the Ethics Committee of the University of Würzburg, and was in accordance with the Declaration of Helsinki.

The frequency of ex1c-SNP A-allele was 9,4%. As there were no subjects with the rare A/A genotype the A/G genotype was compared to the G/G genotype. Regarding ex1f-VNTR, frequencies were: 19% S/S, 50% S/L and 31% L/L, which corresponds to previously described allelic frequencies. Genotypes of both polymorphisms were in Hardy-Weinberg equilibrium (ex1f-VNTR p = 0.92; ex1c-SNP p = 0.08) and genotype groups were not significantly different regarding smoking status, gender distribution, age and education (see supplement Table 1) which was verified via Chi-square tests.

2.2. Genotyping

Ex1f-VNTR polymorphism was determined by PCR amplification and product size determination by means of fragment analysis on a CEQ8000 DNA-sequencer (Beckman-Coulter, Krefeld, Germany), as previously described [10]. Ex1c-SNP was genotyped by standard PCR and subsequent digest with Fnu4HI followed by gel electrophoresis.

2.3. Salivary cortisol

Saliva samples were obtained from each subject at the very beginning and at the end of the test battery and salivary cortisol was measured via Immuno-Assay (biochemical laboratory, TU Dresden) [23]. The time of the day the subjects were tested did not differ significantly between the genotype groups of both polymorphisms (data not shown).

2.4. Neuropsychological examinations

The neurocognitive domains tested in this study were verbal memory, verbal fluency, working memory and verbal IQ for which we used the California Verbal Learning Test (CVLT) [24], the Regensburger verbal fluency (RWT) [25], an n-back task and subtests of the German version of the Wechsler Adult Intelligence Scale III (WAIS-III) [26].

2.5. Statistics

To determine differences in test performance between the genotype groups for each test variable and differences in cortisol levels the appropriate test statistics were applied with the genotype being the independent variable. Variance analyses (ANOVA) were conducted for the CVLT, RWT and WAIS-III. Since the test values in the n-back task were not normally distributed, non-parametric methods (Kruskal Wallis Tests) were used instead. We retrospectively calculated effect sizes on the basis of the respective difference of the means. Cohen's d ranged between 0.005 and 0.62 (mean 0.15). Based on an effect size of 0.15, our sample size with 280 subjects had a power of 0.25 to detect significant differences between A/G vs. G/G genotype carriers of the ex1c-SNP as well as between S/S vs. S/L and L/L genotype carriers of the ex1f-VNTR.

3. Results

Previous findings suggested a poorer outcome in carriers of the short allele of the ex1f-VNTR and the A-allele of the ex1c-SNP when tested for neurocognitive functioning, which is typically impaired in patients suffering from schizophrenia. Indeed, a borderline significant (p = 0.05) difference in performance was shown in the 2-back subtest of the n-back test for the three genotype groups of the ex1f-VNTR. Homozygous carriers of the short allele committed more commission errors than individuals who were heterozygous which in turn committed more commission errors than those homozygous for the long allele suggesting a gene-dose effect (see Table 1). The two genotype groups of the ex1c-SNP differed in their reaction time in the 2-back task, with carriers of the A-allele being faster to respond without compromising their performance, the difference showing a trend significance (p = 0.065). No difference in test performance could be shown for verbal memory and verbal fluency (see supplement Tables 2 and 3). Concerning verbal intelligence, there was a marginally significant difference between the three ex1f-VNTR genotype groups in only one out of seven subtests (see supplement table 4). The most profound difference between the two ex1c-SNP genotype groups was found in baseline cortisol with carriers of the A-allele having a significantly (p = 0.004) lower pre-test cortisol (see Table 2). Cortisol levels decreased in all genotype groups during the 2 h test battery to a post-test cortisol level that no longer differed significantly.

Table 1
Results of the n-back task for all three ex1f-VNTR genotype groups and for the two ex1c-SNP genotype groups; results are expressed as mean ± SEM; in bold: the significant difference in mean commission errors in the 2-back task with homozygous carriers of the short ex1f-VNTR allele committing more commission errors than heterozygous individuals which in turn committed more commission errors than those homozygous for the long allele; also in bold: the marginally significant difference in reaction time in the 2-back task between the two ex1c-SNP genotype groups with carriers of the A-allele being faster to respond.

ex1f-VNTR genotype	n	1-back Task				2-back Task				3-back Task			
		hits	reaction time [s]	omission errors	commission errors	hits	reaction time [s]	omission errors	commission errors	hits	reaction time [s]	omission errors	commission errors
S/S	46	11.2 ± 0.3	0.49 ± 0.02	0.6 ± 0.2	0.1 ± 0.05	9.9 ± 0.3	0.62 ± 0.02	1.8 ± 0.3	1.0 ± 0.1	7.8 ± 0.3	0.73 ± 0.03	3.9 ± 0.3	1.2 ± 0.2
S/L	123	11.3 ± 0.1	0.52 ± 0.01	0.6 ± 0.1	0.2 ± 0.04	9.7 ± 0.2	0.61 ± 0.01	2.2 ± 0.1	0.9 ± 0.1	7.5 ± 0.2	0.76 ± 0.02	4.4 ± 0.2	1.4 ± 0.2
L/L	77	11.2 ± 0.2	0.51 ± 0.01	0.6 ± 0.1	0.2 ± 0.05	9.6 ± 0.2	0.6 ± 0.02	2.2 ± 0.2	0.6 ± 0.1	7.8 ± 0.3	0.7 ± 0.02	4.0 ± 0.3	0.9 ± 0.1
Kruskal-Wallis H (df = 2)		0.408, $p = 0.815$	1.829, $p = 0.401$	0.606, $p = 0.738$	0.255, $p = 0.880$	1.583, $p = 0.453$	0.887, $p = 0.642$	2.175, $p = 0.337$	**5.982, $p = 0.05$**	0.951, $p = 0.621$	2.190, $p = 0.335$	1.406, $p = 0.495$	2.310, $p = 0.315$

ex1c-SNP genotype	n	1-back Task				2-back Task				3-back Task			
		hits	reaction time [s]	omission errors	commission errors	hits	reaction time [s]	omission errors	commission errors	hits	reaction time [s]	omission errors	commission errors
G/G	202	11.4 ± 0.1	0.52 ± 0.01	0.6 ± 0.1	0.2 ± 0.03	9.9 ± 0.1	0.62 ± 0.01	2.1 ± 0.1	0.9 ± 0.1	7.6 ± 0.2	0.75 ± 0.02	4.4 ± 0.2	1.3 ± 0.1
A/G	48	10.9 ± 0.4	0.48 ± 0.02	0.6 ± 0.2	0.1 ± 0.05	9.2 ± 0.4	0.57 ± 0.03	2.3 ± 0.3	0.7 ± 0.1	7.8 ± 0.4	0.7 ± 0.03	3.7 ± 0.3	1.0 ± 0.2
Kruskal-Wallis H (df = 1)		0.647, $p = 0.421$	1.160, $p = 0.281$	0.028, $p = 0.868$	0.841, $p = 0.359$	1.8, $p = 0.18$	**3.405, $p = 0.065$**	0.394, $p = 0.53$	1.903, $p = 0.168$	0.430, $p = 0.512$	0.817, $p = 0.366$	2.068, $p = 0.15$	0.410, $p = 0.522$

Table 2
Mean values ± SEM for baseline cortisol, post-test cortisol and the difference in pre- and post-test cortisol in all three ex1f-VNTR genotype groups and the two ex1c-SNP genotype groups; in bold: the significant difference in baseline cortisol between the two ex1c-SNP genotype groups with carriers of the A-allele having a significantly lower pre-test cortisol.

ex1f-VNTR genotype	n	baseline cortisol [nmol/l]	n	post-test cortisol [nmol/l]	n	delta cortisol [nmol/l]
S/S	51	10.1 ± 1.1	47	5.7 ± 0.5	47	4.9 ± 1.0
S/L	130	10.1 ± 0.7	124	6.0 ± 0.5	124	4.1 ± 0.5
L/L	83	9.6 ± 0.8	73	5.9 ± 0.5	73	4.1 ± 0.7
F (df = 2)		0.134, $p = 0.875$		0.075, $p = 0.928$		0.361, $p = 0.698$

ex1c-SNP genotype	n	baseline cortisol [nmol/l]	n	post-test cortisol [nmol/l]	n	delta cortisol [nmol/l]
G/G	217	10.3 ± 0.5	201	6.1 ± 0.3	201	4.5 ± 0.4
A/G	51	7.8 ± 0.7	47	5.1 ± 0.5	47	2.9 ± 0.6
T		**(df = 118.877) 2.914, $p = 0.004$**		(df = 246) 1.408, $p = 0.161$		(df = 246) 1.668, $p = 0.097$

4. Discussion

In this study we sought to determine whether two polymorphisms influencing *NOS1* expression have an influence on salivary cortisol and on various subsections of cognition in healthy subjects. In line with previous studies [12], our data suggests an influence of the ex1f-VNTR genotype on working memory performance since carriers of the risk allele committed more commission errors in the 2-back task. As the rate of commission errors is however also a measure for impulsive behavior, this suggests that the polymorphism exerts its influence on working memory performance mediated by an effect on impulsivity as it has been suggested by several previous studies from our and other laboratories [13–15,17,27,28]. The fact that the difference in error rate occurred only in the 2-back task can be explained by the higher discriminatory power of a moderately difficult test that has an adequate item difficulty as opposed to tests that are too easy or too hard [29]. The same goes for the difference in reaction time between the two ex1c-SNP genotype groups. At first glance the fact that carriers of the risk allele are faster to respond correctly seems counterintuitive given that schizophrenic patients carrying the risk allele showed a slower reaction time in this very task. However it has been suggested that, while risk alleles generally increase the likelihood of developing a certain clinical phenotype such as schizophrenia, they may manifest as a behavioral phenotype with adaptive advantages if the threshold to disease is not exceeded [30]. It seems plausible that this advantageous phenotype could not manifest our previous study due to the much smaller sample size of the healthy control sample in this neuroimaging study (A/G + A/A n = 12; G/G n = 16) [21]. The so-called heterozygote advantage, which proposes a higher fitness of heterozygous subjects over either homozygous group, might also explain our observations. However, given that none of our subjects were homozygous for the A-allele we could not verify this hypothesis. Regarding verbal intelligence, a borderline significant difference in test performance could be shown for ex1f-VNTR for one subtest only. An association of *NOS1* with verbal intelligence has previously been reported and while our data weakly supports these findings, it does not provide a sound basis for proposing one subtest as the driving force behind said difference. While we clear our hypotheses for the behavioral part of our study, the issue of multiple testing has to be considered when interpreting the above findings.

Based in literature on *NOS1* knockout animals, we also investigated the effect of *NOS1*-genotypes in the stress response. The lower baseline cortisol in carriers of the risk A-allele of the ex1c-SNP however is at odds with rodent studies, where *NOS1* knockout mice had higher baseline cortisol than wild type mice. A relevant discrepancy between experiment designs however was that higher baseline cortisol in *NOS1* knockout mice was measured when animals were calm and relaxed, while our human subjects already experienced stress at the very beginning of the testing when they did not know what to expect. Their lower "baseline" cortisol may therefore actually reflect blunted cortisol response when faced with a stressful situation; this hypothesis is worthwhile to be followed up in further studies. The rather unexpected decrease of cortisol levels in turn could be a combined result from the diurnal variation over the 2 h timespan of our test battery and the subjects acclimatizing to the test situation.

With regards to the limitations of our study, our collective of subjects was very homogenous regarding age and level of education, reducing variance in the data. It is thus conceivable that the sensitivity of the CVLT, RWT and WAIS-III was not sufficient to show a significant difference in test performance. Based on an effect size of 0.15 derived from retrospective power analysis, our sample size with 280 subjects only had a power of 0.25 to detect significant differences and thus was too small. Given the sample size limitation mentioned above, our study can be deemed preliminary and should be repeated with a larger sample size.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.04.003.

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